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Plasmin is not protective in experimental renal interstitial fibrosis[1]

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Plasmin is not protective in experimental renal interstitial fibrosis.

Background. The plasminogen-plasmin system has potential beneficial or deleterious effects in the context of renal fibrosis. Recent studies have implicated plasminogen activators or their inhibitors in this process.

Methods. The development of renal interstitial fibrosis was studied in mice genetically deficient in plasminogen (*plg*−/− mice) and littermate controls (*plg*+/+ mice) by inducing unilateral ureteric obstruction (UUO) by ligating the left ureter.

Results. Collagen accumulation in the kidney was decreased in *plg*−/− mice at 21 days compared with *plg*+/+ mice by hydroxyproline assay (*plg*+/+ 19.0 ± 1.2 μg collagen/mg tissue, *plg*−/− 15.6 ± 0.5 μg collagen/mg tissue, $P = 0.04$). Macrophage accumulation in *plg*−/− mice was reduced at 21 days, consistent with a role for plasmin in macrophage recruitment in this model. Myofibroblast accumulation, assessed by the expression of α-smooth muscle actin (α-SMA), was similar in both groups at both time points. Endogenous plasmin played a role in the activation of transforming growth factor-β (TGF-β), as *plg*−/− mice had lower ratios of βig-h3:TGF-β1 mRNA than *plg*+/+ mice. Matrix metalloproteinase (MMP)-9 activity was unchanged in the absence of plasmin, but MMP-2 activity was decreased.

Conclusion. Plasminogen, the key proenzyme in the plasminogen-plasmin system, does not protect mice from experimental interstitial fibrosis and may have significant pathogenetic effects. These findings, together with other recently published studies in the biology of renal fibrosis, imply that effects of proteins such as plasminogen activator inhibitor-1 (PAI-1), tissue-type plasminogen activator (tPA), and urokinase-type plasminogen activator receptor (uPAR) on renal fibrosis occur independently from the generation of plasmin.

Interstitial fibrosis is an important common pathway to end state renal disease. The infiltration of macrophages, the presence of interstitial cells expressing α-smooth muscle actin (α-SMA) and laying down of interstitial matrix proteins including collagen are prominent features of this process [1]. A number of factors, including inflammatory cells, the activity of tubular cells, the accumulation of myofibroblasts, the secretion of soluble factors and net balance between deposition and turnover of matrix proteins are thought to influence the rate of progression of fibrosis.

The plasminogen-plasmin system performs important intravascular roles via the conversion of fibrin to fibrin degradation products by plasmin. Plasmin is generated by conversion of its proenzyme, plasminogen to plasmin by either tissue-type plasminogen activator (tPA) or urokinase-type plasminogen activator (uPA). Plasminogen is the only known substrate for plasmin and mice deficient in plasminogen are totally plasmin deficient [2]. The similar phenotype and absence of plasmin activity of mice deficient in either plasminogen, or both tPA and uPA has demonstrated that tPA and uPA are the only physiologically relevant activators of plasminogen in vivo [2, 3]. tPA is the major glomerular plasminogen activator, while uPA is more prominently expressed in the renal tubulointerstitium [4, 5]. In addition to direct effects on soluble plasminogen, uPA can act via a cell-associated receptor, the uPA receptor (uPAR). Both tPA and uPA are inhibited by plasminogen activator inhibitors (PAIs), the best known being PAI-1. Studies in fibrin-related/dependent glomerular disease have shown that endogenous plasmin limits glomerular fibrin accumulation [6]. However, the role of the plasminogen-plasmin system in renal fibrosis is less clear. Plasmin activity is increased approximately threefold in hydronephrotic mouse kidneys, with the increase being apparent by day 3 [7]. Studies using the same model as the current studies showed that despite similar renal plasmin activity mice deficient in PAI-1 or tPA showed protection from disease [7, 8], while uPAR-deficient mice demonstrated accelerated fibrosis [9]. These results suggest roles for PAI-1, tPA, and uPAR that might be independent from their actions to inhibit or facilitate the conversion of plasminogen to plasmin. However, experimental fibrosis in other organs, including the

[1] **See Editorial by Zheng and Harris, p. 455.**

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lungs and liver [10, 11], is accelerated in the absence of plasminogen.

Plasmin, by virtue of its ability to directly degrade matrix and activate matrix metalloproteinases (MMPs), could have a protective role in limiting collagen and matrix accumulation in renal fibrosis. However, plasmin has the capacity to play pathogenetic roles in renal disease via its potential role in leukocyte recruitment [12] or by converting transforming growth factor-β (TGF-β), a growth factor that plays a pathogenic role in renal fibrosis [13–15] to its active form. Plasmin may directly activate latent TGF-β or release latent TGF-β from storage sites within matrix [16]. Plasmin also has the potential to activate MMPs that could participate in tubular basement membrane degradation [8]. The availability of plasminogen-deficient mice (*plg*−/− mice) [2] that are totally deficient in plasmin but have normal levels of tPA, uPA, and PAI-1 [17] allows examination of the role of endogenous plasmin in fibrotic renal injury. These studies address the hypothesis that in experimental renal interstitial fibrosis, endogenous plasmin is protective. They use mice that are plasminogen deficient (*plg*−/− mice) and littermate controls (*plg*+/+) in which experimental unilateral ureteric obstruction (UUO) or ligation has been induced. Fibrotic renal disease in the obstructed kidney was studied at an early time point (7 days after ureteric ligation) and a later time point (21 days after ligation).

METHODS

Experimental design

Mice with a genetic deletion of plasminogen (*plg*−/−) [2] on a C57BL/6 × 129/SvJ background, backcrossed once onto a C57BL/6 background (75% C57BL/6 and 25% 129/SvJ) were bred in an SPF facility (Leuven, Belgium) by intercrossing *plg*+/− males and females. Offspring were genotyped and *plg*−/− mice, all carrying two copies of the disrupted allele and littermate control *plg*+/+ mice were identified. Mice aged 8 to 12 weeks were used for experiments. All mice in experiments at 7 days were male; mice studied at 21 days were female. The left ureter of each mouse was ligated under general anesthesia. Renal injury was studied at 7 days and at 21 days after UUO. Histologic assessment was performed on coded slides. Results are expressed as the mean ± SEM. The significance of differences between groups was determined by Mann-Whitney *U* test (GraphPad Prism, GraphPad Software Inc., San Diego, CA, USA). The following two groups of mice were studied: (*1*) *plg*+/+ mice, UUO day 7 ($N = 8$), UUO day 21 ($N = 9$); and (*2*) *plg*−/− mice, UUO day 7 ($N = 7$), UUO day 21 ($N = 8$).

Assessment of interstitial fibrosis and collagen accumulation

Kidney tissue was fixed in 2% paraformaldehyde, embedded in paraffin, and 3 μm tissue sections from all mice were stained with picro-Sirius red (Sigma Chemical Co., St. Louis, MO, USA) identifying collagen fibers (in red) [18]. Different sections from the same mice were also stained with Masson trichrome identifying collagen fibers (in blue). Histologic assessment of collagen deposition was determined by the point counting method using a 10 × 10 grid. A minimum of 10 high power (×400) fields were assessed per animal and results expressed as % total interstital cortical area, excluding glomeruli, blood vessels, and periglomerular and perivascular areas [19]. Biochemical measurement of renal collagen content was assessed by determining total hydroxyproline according to the method of Bergman and Loxley [20]. An accurately weighed portion of renal tissue (one fourth of the obstructed or contralateral kidney) was hydrolysed in 6 N HCl by incubation at 110°C overnight. The hydrolysate was neutralized with 2.5 mol/L NaOH. Hydrolysates in isopropanol were oxidised by chloramine T then mixed with p-dimethylaminonbenzaldehyde (25 minutes, 60°C) and the absorbance measured at 558 nm. Total collagen was calculated using the assumption that collagen contains 12.7% hydroxyproline by weight. Results were expressed as μg/mg kidney wet weight.

Interstitial macrophage accumulation

Kidney tissue was fixed in periodate lysine paraformaldehyde (PLP) for 4 hours, washed in 7% sucrose solution, then frozen in liquid nitrogen. Tissue sections (6 μm) were stained to demonstrate macrophage infiltrate with F4/80 using a three-layer immunoperoxidase technique, as previously described [21, 22]. As individual macrophages could not be reliably counted, a minimum of 10 high power fields were assessed per animal and cortical interstitial infiltrate scored using a scale of 0 to 3+ [0, normal (equivalent to normal animals and the contralateral kidney of experimental animals); 1, 10% to 40% interstitium; 2, 40% to 70% interstitium; and 3, >70% interstitium].

Renal myofibroblast accumulation

Interstitial myofibroblasts were assessed by detecting α-SMA by immunohistochemistry and Western blotting. For immunohistochemistry, paraformaldehyde-fixed tissue sections (3 μm) were stained with peroxidase-conjugated murine antihuman α-SMA 1A4 monoclonal antibody using the enhanced polymer one-step staining (EPOS) reagent (Dako Corp., Carpinteria, CA, USA). Briefly, α-SMA antibody was incubated overnight at 4°C and antibody binding detected using 3,3′-diaminobenzidine (DAB) as the chromogen (Sigma Chemical Co.) and counterstained with nuclear fast red (BDH Chemicals, Poole, England). Dako EPOS immunoglobulins-horseradish peroxidase (HRP)–specific negative control was used to control for any nonspecific staining. Interstitial α-SMA accumulation was assessed by the point counting method (as above).

For Western blotting, kidney samples (four mice per group at each time point) were homogenized in protein extraction buffer (0.01 mol/L $CaCl_2$ and 0.25% Triton X-100). The samples were centrifuged at 13000*g*, 4°C for 30 minutes. Supernatant collected and protein concentrations were determined using the Bradford protein assay. Protein samples (10 μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and transferred to a nitrocellulose membrane. Membranes were stained with mouse anti-α-SMA antibody (1:1000, 1A4) (Dako) or mouse-anti-β-tubulin antibody (1:1000, D-10) (Pierce, Rockford, IL, USA) and followed by goat anti-mouse IgG conjugated to HRP (1:10000, Pierce). Detection was performed using SuperSignal West Pico Chemiluminescent substrate according to the manufacturer's recommendations (Pierce). α-SMA expression was analyzed using Image Gauge (Science Lab, Fuji Photo Film, Tokyo, Japan) and results expressed as the ratio to β-tubulin.

Assessment of intrarenal TGF-β1 and TGF-β-inducible gene-h3 (βig-h3) mRNA in obstructed kidneys

To assess the potential for plasmin to activate TGF−β, TGF-β1, and βig-h3 (a TGF-β–induced gene product [23]) mRNA were assessed in obstructed kidneys from *plg*+/+ (UUO day 7, $N = 4$; UUO day 21, $N = 4$) and *plg*−/− mice (UUO day 7, $N = 3$; UUO day 21, $N = 4$) with UUO. Frozen mouse kidney tissue, stored at −80°C was homogenized, total RNA was isolated using TRIzol reagent (Life Technologies, Grand Island, NY, USA), and purified RNA was dissolved in sterile water and quantified spectrophotometrically. RNA quality was verified on a 1% denaturing agarose gel by visualizing the 18S and 28S RNA bands. Four micrograms of total RNA was treated with RQ1 DNase (1 U/μL) (Promega, Madison, WI, USA) to remove genomic DNA. The DNase treated RNA was reverse transcribed with 1 μL (2 μg/μL) random hexamers (Roche Diagnostics, Mannheim, Germany) and incubated for 5 minutes at 70°C. After cooling on ice for 5 minutes, 5 μL of 5× avian myeloblastosis virus (AMV) reaction buffer, 2.5 μL of 10 mmol/L deoxynucleoside triphosphate (dNTP) mix, 0.5 μL RNase inhibitor (40 U/μL) (Roche Diagnostics), 0.5 μL AMV reverse transcriptase (25 U/μL) (Roche Diagnostics) and 4.5 μL of diethyl pyrocarbonate (DEPC) water was added. The reaction mixture was incubated at 37°C for 60 minutes to complete cDNA synthesis, then cDNA samples were stored at −20°C for future use.

TGF-β1 and βig-h3 gene expression were measured and quantified using ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Sequence specific primers were designed to span exon-exon boundaries to avoid amplification of contaminating genomic DNA using the Primer Express software, version 1.5 (Applied Biosystems) (Table 1). Primers were obtained from Sigma-Aldrich and fluorescent probes from Applied Biosystems.

Table 1. Sequences for analysis of mRNA for transforming growth factor-β1 (TGF-β1) and βig-h3 by real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR)

	Sequence
TGF-β1	
Forward primer	AGAAGTCACCCGCGTGCTA
Reverse primer	TGTGTGATGTCTTTGGTTTTGTCA
Probe	TGGTGGACCGCAACAACGCAAT
βig-h3	
Forward primer	TCCTTGCCTGCGGAAGTG
Reverse primer	GGAGAGCATTGAGCAGTTCGA
Probe	TGGACTCCCTGGTGAGCAACGTCAAC

For the relative quantification of the target gene and the endogenous control 18S ribosomal RNA (18S), real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed using the TaqMan assay which utilizes the 5′ nuclease activity of DNA polymerase to hydrolyze a fluorescence probe bound to its target amplicon. A TaqMan probe contains a fluorescence reporter dye [6-carboxyfluorescein (FAM)] at the 5′-end and a fluorescent quencher [6-carboxytetramethylrhodamine (TAMRA)] at the 3′-end. A commercial, predeveloped (18S) control kit labeled with the fluorescent reporter dye (VIC) on the 5′ end, and the quencher (TAMRA) on the 3′ end (Applied Biosystems) was used as the endogenous control.

Amplification efficiencies of the target genes, and the endogenous control, 18S were approximately equal as demonstrated by validation experiments (data not shown) allowing relative quantitation to be performed between the target gene and endogenous control. Primer and probe concentrations were optimized before experiments were completed. The 25 μL PCR mixture contains 12.5 μL of TaqMan Univeral PCR Master Mix, 500 nmol/L primers (forward and reverse), 100 nmol/L of TaqMan probe, and 1 μL of cDNA template. PCR was performed at 50°C for 2 minutes, 95°C for 10 minutes to activate the AmpliTaq Gold polymerase (Applied Biosystems), then run for 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Experiments were performed in triplicate for each sample and no template controls were added to ensure that amplification was not due to contamination of other components within the PCR mixture. Data analysis was performed using the Applied Biosystems Comparative C_T method (*http://www.appliedbiosystems.com.au/sdscompendiums.htm*).

MMP-2 and MMP-9 activation in the absence of plasminogen

Gelatin zymography was performed to analyze activated MMP-2 and MMP-9 as previously described [24]

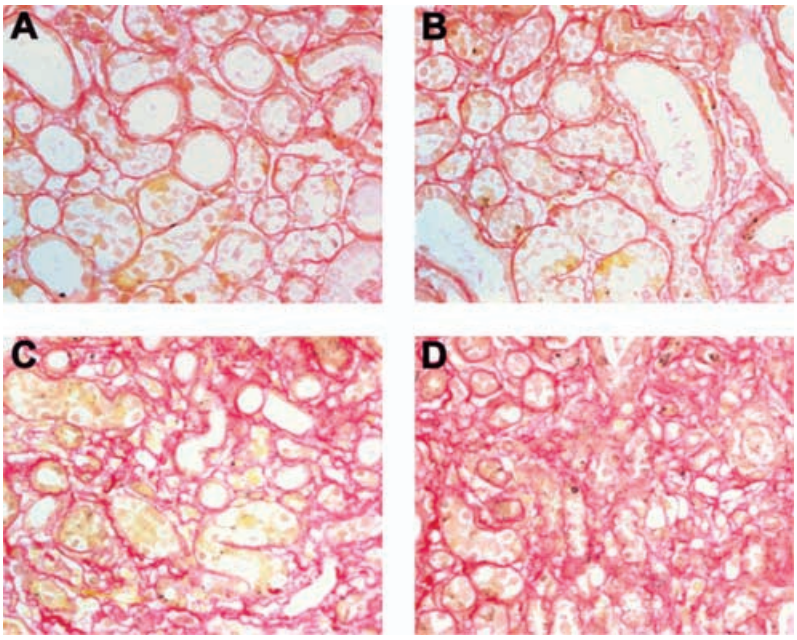

Fig. 1. Renal histopathology in *plg*+/+ and *plg*−/− mice. Interstitial renal fibrosis developed in both plasminogen-intact and plasminogen-deficient mice. Disease was moderate at day 7 in both *plg*+/+ mice (*A*) and *plg*−/− mice (*B*), and severe at day 21, again in both *plg*+/+ mice (*C*) and *plg*−/− mice (*D*) (high power, ×400, Sirius red stain, collagen appearing red).

using four mice from each time point. Samples (10 μg protein/well) were loaded onto 10% SDS-polyacrylamide gels containing 1 mg/mL gelatin as substrate (Bio-Rad Laboratories, Hercules, CA, USA). Human MMP-2 and MMP-9 (a gift from Dr L.A. Salamonsen, Melbourne, Australia) were used as standards. After protein separation by electrophoresis under nonreducing conditions, the gel was washed in 0.05 mol/L Tris buffer pH 7.4 (containing 5 mmol/L $CaCl_2$, 1 μmol/L $ZnCl_2$, 0.01% NaN_3, and 0.25% Triton X-100) for 30 minutes at room temperature then in Tris buffer without Triton X-100 for 15 minutes at room temperature before incubating at 37°C for 48 hours. The gel was stained with Coomassie blue (Bio-Rad Laboratories) and pro-MMPs and active MMPs were visualized by negative staining at the appropriate molecular weights. Each lytic band was analyzed using Image Gauge and results expressed as arbitrary units (AU).

RESULTS

Endogenous plasminogen and plasmin does not protect mice from renal interstitial fibrosis or collagen accumulation

Obstructed kidneys from genetically normal *plg*+/+ mice developed progressive renal cortical thinning and fibrosis from day 7 to day 21 of disease, compared with contralateral kidneys (obstructed kidneys day 7, Fig. 1A) (obstructed kidneys day 21, Fig. 1C). However, renal interstitial fibrosis was not accelerated in the absence of plasmin (day 7, Fig. 1B; day 21, Fig. 1D). Histologic assessment by point counting of Sirius red–stained sections and Masson trichrome–stained sections to derive the percentage of cortical interstitial section staining for collagen showed a similar degree of progressive renal fibrosis in obstructed kidneys from both *plg*+/+ and *plg*−/− mice (Table 2). Quantitation of renal collagen accumulation (Fig. 2) by hydroxyproline assay showed similar degrees of collagen content in the contralateral (unobstructed) and normal kidneys (4.6 ± 0.2 μg collagen/mg kidney). In obstructed kidneys, there was no difference between *plg*+/+ and *plg*−/− mice at 7 days (*plg*+/+ 7.6 ± 0.4 μg collagen/mg kidney and *plg*−/− 7.6 ± 0.3 μg collagen/mg kidney) (Fig. 2A). At 21 days, not only did endogenous plasmin not provide any protection from the development of renal fibrosis, renal collagen accumulation was significantly *less* in mice genetically deficient in plasminogen, and therefore plasmin [*plg*+/+ 19.0 ± 1.2 μg collagen/mg kidney and *plg*−/− 15.6 ± 0.5 μg collagen/mg kidney ($P = 0.04$)] (Fig. 2B).

Table 2. Histologic assessment of collagen accumulation using both Sirius red staining and Masson trichrome staining, by point counting (10 × 10 grid) of a minimum of 10 high power fields per animal, expressed as % total interstitial cortical area, excluding glomeruli, blood vessels, and periglomerular and perivascular areas

		Sirius red	Masson trichrome
Day 7			
Obstructed	*plg*+/+	14.0 ± 2.7	11.0 ± 2.9
	plg−/−	8.9 ± 1.3	27.4 ± 7.6
Day 21			
Obstructed	*plg*+/+	78.5 ± 4.7	65.5 ± 5.6
	plg−/−	82.3 ± 3.2	55.8 ± 5.6

P values for *plg*+/+ vs. *plg*−/− mice at day 7 were $P = 0.12$ for Sirius red staining and $P = 0.13$ for Masson trichrome staining (Mann-Whitney *U* test).

Macrophage recruitment is diminished in the absence of plasminogen

Plasmin has the capacity to promote macrophage recruitment into inflamed tissues [12]. Immunostaining

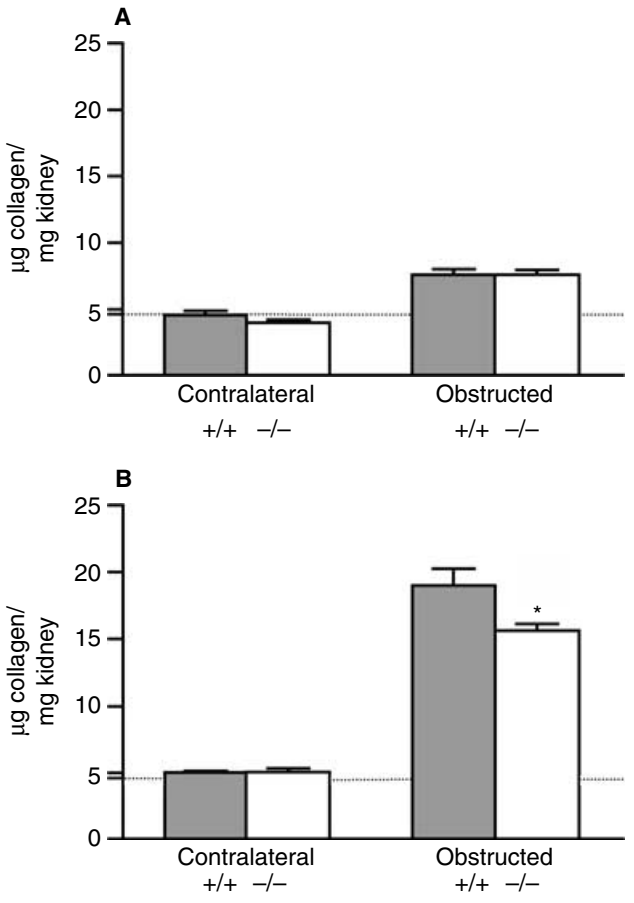

Fig. 2. Accumulation of collagen in normal contralateral and obstructed kidneys from *plg*+/+ and *plg*−/− mice. Hydroxyproline assay demonstrates no difference at 7 days (*A*) and a small but significant reduction in collagen accumulation at day 21 (*B*) $^*P = 0.04$. The dotted lines represent values for unobstructed normal mice on which operations have had not been performed.

using the macrophage specific monoclonal antibody F4/80 showed that macrophages were present in both *plg*+/+ and *plg*−/− mice at 1 week (Fig. 3A, B, and E) but the macrophage infiltrate was diminished in the interstitium of *plg*−/− mice (Fig. 3D and E) compared with *plg*+/+ mice at 3 weeks (Fig. 3C and E).

Interstitial myofibroblast accumulation is not affected by plasminogen deficiency

Tubular cells undergo transdifferentiation into myofibroblasts under inflammatory stimuli and participate in fibrosis. Myofibroblasts can be identified by their expression of α-SMA. Therefore α-SMA was used as a surrogate marker for myofibroblast accumulation and assessed by Western blotting and point counting of immunohistologic sections. Assessed by point counting of α-SMA–positive areas in the cortical interstitium, there was no difference in the accumulation of myofibroblasts between *plg*+/+ mice and *plg*−/− mice (day 7, *plg* +/+ 41.0% ± 3.4% of the sections involved and *plg*−/− 48.1% ± 7.5%; day 21, *plg*+/+ 43.9% ± 2.8% and *plg*−/− 40.5% ± 3.0%). Similarly, semiquantitative assessment α-SMA protein by Western blotting revealed no differences between *plg*+/+ and *plg*−/− mice [day 7, *plg*+/+ 0.26 ± 0.09 (α-SMA:β-tubulin ratio) and *plg*−/− 0.42 ± 0.09 ($P = 0.63$); day 21, *plg*+/+ 0.63 ± 0.07 and *plg*−/− 0.59 ± 0.20 ($P = 0.49$)].

The βig-h3 to TGF-β1 mRNA ratio is diminished in plasminogen-deficient mice with UUO

To assess whether plasmin is likely be involved in the activation of TGF-β in vivo, mRNA for TGF-β1 and βig-h3 (a TGF-β responsive gene [23]) were measured in obstructed kidneys from *plg*+/+ and *plg*−/− mice (Fig. 4). There was a good correlation between levels of mRNA for TGF-β1 and βig-h3 in both *plg*+/+ mice ($r^2 = 0.76$) and *plg*−/− mice ($r^2 = 0.84$). The ratio of βig-h3:TGF-β1 was lower in *plg*−/− mice at both time points, with the difference approaching statistical significance ($P = 0.057$ for both day 7 and day 21).

MMP-2 and MMP-9 in fibrotic kidneys from *plg*+/+ and *plg*−/− mice

MMPs have the capacity to be activated by plasmin and may play a role in matrix degradation. Gelatin zymography was performed on kidneys from *plg*+/+ and *plg*−/− mice at both 1 and 3 weeks. Active and pro-MMP-9 were unchanged in the absence of plasminogen (Table 3) (Fig. 5). Active MMP-2 levels in contralateral kidneys from either strain were too low to be reliably detected (Table 4) (Fig. 5). Levels rose to a similar degree in both *plg*+/+ and *plg*−/− mice at week 1, but were lower in the absence of plasminogen at 3 weeks [*plg*+/+ 46.2 ± 1.4 AU and *plg*−/− 25.1 ± 2.1 AU ($P = 0.03$)]. This reduction in active MMP-2 in obstructed kidneys in the absence of plasminogen was not a result of reduced total MMP-2, as analysis of pro-MMP-2 and active MMP-2 levels showed no reduction in total MMP-2 in *plg*−/− mice [*plg*+/+ 93.8 ± 4.2 AU and *plg*−/− 83.1 ± 7.7 AU ($P = 0.34$)].

DISCUSSION

These studies demonstrate for the first time that plasmin is not protective in renal interstitial fibrosis, and provide some evidence that plasmin may in fact be pathogenetic. As plasmin promotes matrix remodelling, it has been assumed that endogenous plasmin would play a protective role in renal interstitial fibrosis, by limiting collagen and other matrix accumulation. However, this hypothesis (that plasmin would be protective in renal fibrosis) has not been tested. Plasmin activity is increased threefold in murine UUO [7], while recent studies in mice deficient in some of the other proteins that play a role in the plasminogen-plasmin system, namely PAI-1 [7], tPA [8], and uPAR [9], have shown pathogenetic roles (in the case of tPA perhaps somewhat unexpectedly) for PAI-1

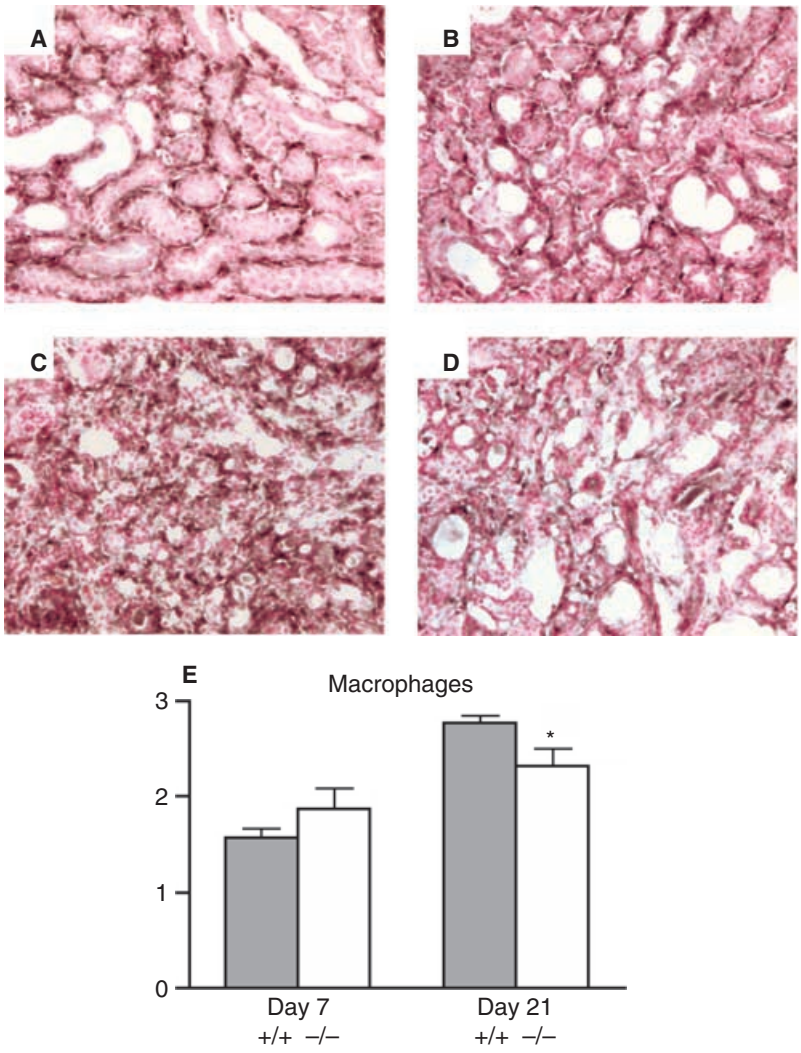

Fig. 3. Macrophage infiltrate in obstructed kidneys from *plg*+/+ and *plg*−/− mice. Photomicrographs of interstitial macrophages. At 7 days there was a moderately dense macrophage infiltrate in both *plg*+/+ (*A*) and *plg*−/− (*B*). At 21 days, the infiltrate in *plg*+/+ mice (*C*) was more dense than in *plg*−/− mice (*D*) (medium power, ×200, F4/80+ cells, black reaction product, nuclear fast red counterstain). (*E*) Semiquantitative analysis of macrophage infiltration, showing reduced infiltration at 21 days in mice deficient in plasminogen.

and tPA, and a protective role for uPAR in the pathogenesis of renal fibrosis. These changes seemed to have occurred independent of plasmin generation, in the case of PAI-1 potentially by direct effects on macrophages and myofibroblasts [7], with tPA by promoting basement membrane degradation and enhancing tubular epithelial to myofibroblast transformation [8], while the protective effects of uPAR are possibly due to enhanced degradation of mediators (such as PAI-1) that are profibrotic [25]. These recent studies add weight to the question answered in these studies—is plasmin itself protective in renal interstitial fibrosis?

Mice deficient in plasminogen are also deficient in plasmin and plasmin activity and since their generation in 1995 have been used in several systems to test the role of plasmin in normal and pathologic processes [2, 6, 26–28]. Using a model of renal interstitial fibrosis induced by UUO, our studies find that fibrosis is not accelerated in the absence of plasmin, at two time points, 7 days and 21 days. Quantitative measurements of collagen accumulation by hydroxyproline assay showed no increase in renal collagen content at 1 week and a small but statistically significant *decrease* in renal collagen content in *plg*−/− mice, that lack plasmin, at 3 weeks. There were no significant differences in fibrosis in histologically stained sections assessed by point counting, which may reflect differences between assessing renal collagen content biochemically and histologically by considering the presence or absence of collagen in each individual point of a 10 × 10 grid. Therefore, endogenous plasmin does not protect from interstitial fibrosis, and in fact may have deleterious effects.

Plasminogen-plasmin has the capacity to recruit macrophages via the binding of plasminogen to annexin II [29]. In our studies, UUO resulted in a dense interstitial infiltrate of macrophages that could not be accurately individually counted but could be semiquantitatively assessed by examining the degree of infiltrate in a number of high powered fields from each animal. While no difference could be detected at 1 week, at 3 weeks, the time point at which there was more collagen accumulation, obstructed kidneys from *plg*−/− mice had a less dense

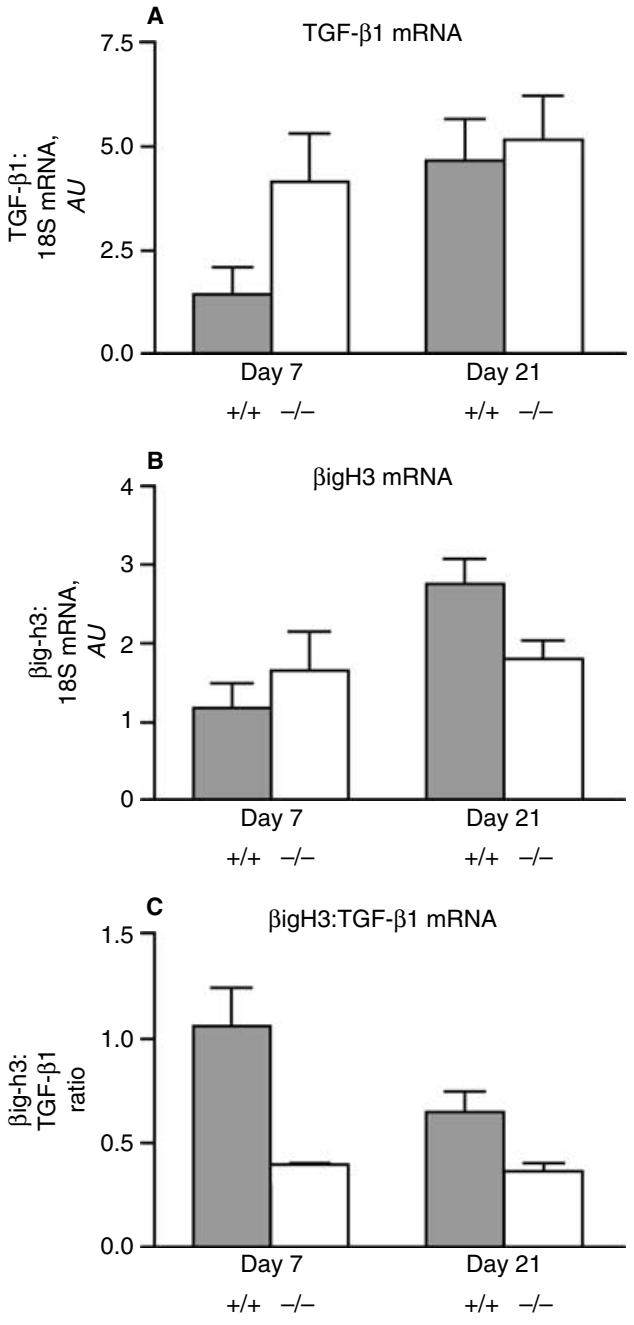

Fig. 4. Analysis of transforming growth factor-β1 (TGF-β1) and βig-h3 mRNA in obstructed kidneys from *plg*+/+ and *plg*−/− mice at 7 and 21 days. TGF-β1 and βig-h3 mRNA are compared with the housekeeping gene 18S, with the βig-h3:TGF-β1 mRNA ratio providing an index of active to total TGF-β. Results are expressed in arbitrary units (AU) relative to *plg*+/+ mice at day 7. (*A*) There was a trend, but no significant difference ($P = 0.114$) to increased TGF-β1 mRNA at day 7, that was not continued at day 21. (*B*) There was no significant reduction in βig-h3 mRNA ($P = 0.11$ at 21 days). (*C*) There was a strong trend ($P = 0.057$ at either time point) to reduced βig-h3:TGF-β1 mRNA ratios in *plg*−/− mice.

Table 3. Matrix metalloproteinase (MMP)-9 in *plg*+/+ and *plg*−/− mice with unilateral ureteral obstruction (UUO)

		Pro-MMP-9	MMP-9
Day 7			
Contralateral	*plg*+/+	51.2 ± 8.5	79.0 ± 31.0
	plg−/−	54.7 ± 13.9	52.8 ± 12.4
Obstructed	*plg*+/+	98.7 ± 17.8	103.5 ± 23.0
	plg−/−	54.0 ± 9.3	92.9 ± 17.6
Day 21			
Contralateral	*plg*+/+	21.0 ± 1.4	41.9 ± 10.3
	plg−/−	14.5 ± 1.8	39.3 ± 11.1
Obstructed	*plg*+/+	39.8 ± 11.3	50.0 ± 14.0
	plg−/−	30.8 ± 1.7	40.1 ± 1.1

Levels of MMP-9 were unaltered in the absence of plasminogen. Results are expressed in arbitrary units.

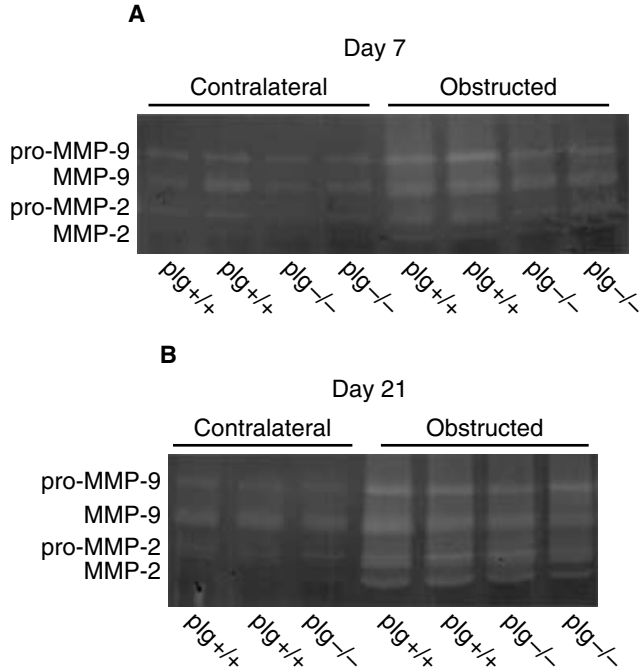

Fig. 5. Gelatin zymography showing pro-matrix metalloproteinase (MMP)-9 and active MMP-9, and pro-MMP-2 and active MMP-2 activity in kidneys from *plg*+/+ and *plg*−/− mice. Pro-MMP-2 but not active MMP-2 activity is measurable in contralateral kidneys and active MMP-2 is increased in obstructed kidneys from *plg*+/+ mice, but to a lesser degree in *plg*−/− mice. Plasmin is not required for MMP-9 activation. Representative samples from each group of mice were chosen.

macrophage infiltrate. This finding may help explain the reduction in renal collagen accumulation in *plg*−/− mice, as evidence is accumulating that macrophages are pathogenetic in renal fibrosis [30]. Recent studies suggest that macrophage depletion, while not affecting interstitial fibroblast accumulation, did reduce collagen deposition [abstract; Kipari, et al, *J Am Soc Nephrol* 13:541A, 2002]. In agreement with this report, our studies found that despite reduced macrophage accumulation, *plg*−/− mice did not have decreased α-SMA–positive interstitial myofibroblasts at either time point studied. However, not all studies have demonstrated that increased macrophage accumulation results in increased collagen deposition. Recent studies in *uPAR*−/− mice [25] and angiotensin II type 1 receptor–deficient (*Agtr*−/−) mice suggest that both activation status and potentially protective functions of macrophages are relevant to fibrosis [31].

Table 4. Matrix metalloproteinase (MMP)-2 in *plg*+/+ and *plg*−/− mice with unilateral ureteral obstruction (UUO)

		Pro-MMP-2	MMP-2
Day 7			
Contralateral	*plg*+/+	44.2 ± 23.9	ND
	plg−/−	25.5 ± 3.7	ND
Obstructed	*plg*+/+	58.8 ± 21.7	11.4 ± 0.9
	plg−/−	76.6 ± 17.4	13.2 ± 1.3
Day 21			
Contralateral	*plg*+/+	20.8 ± 3.9	ND
	plg−/−	14.9 ± 1.2	ND
Obstructed	*plg*+/+	47.6 ± 3.3	46.2 ± 1.4
	plg−/−	57.0 ± 9.4	25.1 ± 2.1[a]

ND is not detectable. Active MMP-2 was so low as to be undetectable in contralateral kidneys but was present in obstructed kidneys and at day 21 was lower in *plg*−/− mice than in *plg*+/+ mice. Results are expressed in arbitrary units.

[a] $P = 0.03$ compared with *plg*+/+ mice (Mann-Whitney U test).

Endogenous plasmin plays an important role in limiting crescentic glomerulonephritis [6], where glomerular fibrin has been shown to be an important mediator of injury [32, 33]. The model used in the current studies is not classically mediated by fibrin, although the definitive functional study of UUO in fibrinogen-deficient mice has yet to be performed. In previous studies in experimental crescentic glomerulonephritis, fibrin was shown to be chemotactic for macrophages [34], and glomerular macrophage accumulation was increased in *plg*−/− mice [6] together with a major increase in glomerular fibrin deposition. In contrast, the current studies found fewer macrophages in the interstitium of *plg*−/− mice, concordant with the findings of Ploplis et al [12], who showed impaired migration of macrophages into the peritoneum of thioglycollate-treated *plg*−/− mice. Assessment of macrophage numbers in glomeruli in our study (although this model is not characterized by prominent macrophage accumulation) showed low numbers (<0.2 per glomerular cross section) of F4/80+ cells and no difference between *plg*+/+ and *plg*−/− mice (data not shown). Differences in macrophage recruitment in the two studies are likely to be due to both the relative contributions of fibrin to the lesions, and to the potential for macrophages in glomeruli to express a different phenotype to those in the interstitium [35].

TGF-β is a key fibrogenic growth factor that has been convincingly implicated in both human and experimental renal interstitial fibrosis [13–15]. It is secreted as a latent form non-covalently associated with the TGF-β propeptide homodimer [16] and requires conversion to an active form. Plasmin is capable of activating TGF-β [16] and in the current studies we have provided evidence that in vivo this occurs. Measuring mRNA for TGF-β1 and for βig-h3, a TGF-β responsive gene [23], mice *plg*−/− mice had an approximately 50% reduction in βig-h3 mRNA when compared with TGF-β1 mRNA. These data suggest that at least in this experimental model of renal fibrosis plasmin is playing a role in converting TGF-β to its active form and highlight a potentially pathogenetic effect of plasmin in renal interstitial fibrosis.

MMPs, secreted as inactive pro-MMPs and later activated, have significant roles in matrix turnover and also in basement membrane degradation. While theoretically increased active MMPs might limit renal collagen and other matrix accumulation, data from studies in both the kidney [8] and in other organs have highlighted to potential injurious roles of MMPs. In the same model used in the current studies, MMP-9 (gelatinase B) was activated by tPA and MMP-9 degraded tubular basement membrane and promoted tubular epithelial cell to myofibroblast transformation [8]. In our studies there were no changes in MMP-9 activity in the absence of plasminogen, confirming that the changes observed in the study of Yang et al [8] were, as their data suggested, plasmin independent. However, in the absence of plasmin, MMP-2 activity was decreased at 21 days. It is not clear whether this is a direct effect of the absence of plasmin, for although one study has suggested that plasmin can activate MMP-2 from human mesangial cells [36], others suggest that murine MMP-2 is not activated by plasmin [37]. It is likely, however, that the reduction in MMP-2 is related to the decrease in activated TGF-β (measured by βig-h3:TGF-β mRNA ratio) observed in these studies, as TGF-β may induce MMP-2 [38]. However, alternative explanations such as activation by a macrophage-derived soluble factor are possible.

Sex hormones have the potential to modify the progression of renal disease [39] and also may influence components of the plasminogen-plasmin system [40]. Our studies used male mice for the 7-day time point and female mice for the 21-day time point and both time points were analyzed separately. It is possible that the similar macrophage and collagen accumulation in male *plg*+/+ and *plg*−/− mice at 7 days might reflect differences between the magnitude of plasmin's effects in males and females, although other explanations, such as the timing of events at different stages of fibrosis, are possible.

Given the potential protective and pathogenetic effects of plasmin, the net effects of plasmin may be protective or harmful depending on the relative contributions of, for example, macrophage accumulation, matrix degradation, TGF-β activation, and MMPs. The effects of inhibiting or enhancing plasmin require careful evaluation in fibrosis mediated by different fibrotic stimuli to establish the role of plasmin in different fibrotic diseases.

CONCLUSION

These studies show that endogenous plasmin is not protective in experimental renal fibrosis, induced by ureteric obstruction. They add significantly to our understanding of the plasminogen-plasmin system in disease by demonstrating that mechanism of fibrosis are organ-specific and that plasmin can have deleterious effects in fibrotic injury.

They demonstrate that pathogenetic effects observed in renal fibrosis using mice deficient in other components of the plasminogen-plasmin system (e.g., tPA and PAI-1) are plasmin independent.

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